Comparative Biochemistry and Physiology, Part C 167 (2015) 140–146

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Purification of a secreted lectin from *Andrias davidianus* skin and its antibacterial activity

Min Qu [a,1], Changqing Tong [a,1], Liang Kong [b], Xin Yan [a], Oleg V. Chernikov [d], Pavel A. Lukyanov [c,d,*], Qiao Jin [a], Wei Li [a,e,**]

[a] College of Food Science and Engineering, Dalian Ocean University, Dalian 116023, PR China
[b] College of Marine Technology and Environment, Dalian Ocean University, Dalian 116023, PR China
[c] College of Life Science, Heilongjiang University, Harbin 150080, PR China
[d] G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Science, Vladivostok 690022, Russia
[e] Jinchi Giant Salamander Biological Technology Co., Ltd, Zhangjiajie (China), Zhangjiajie 427400, PR China

ARTICLE INFO

Article history:
Received 4 June 2014
Received in revised form 4 August 2014
Accepted 11 August 2014
Available online 23 October 2014

Keywords:
Andrias davidianus
Skin secretion
Lectin
Antibacterial activity
Respiration

ABSTRACT

A lectin secreted from *Andrias davidianus* skin (ADL) was purified by affinity chromatography on porcine stomach mucin (type III) (PSM)-crosslinked albumin, followed by gel filtration on Sephadex G-100 and HPLC on TSK gel G3000PW$_{XL}$. The purified lectin was found to be a dimeric protein, as revealed by SDS-PAGE and MALDI-TOF analysis. SDS-PAGE showed that the ADL protein had a molecular mass of 17 kDa. ADL produced an 8.5 kDa band when examined using SDS-PAGE under reducing conditions. ADL agglutinated native and trypsinized human B erythrocytes. The hemagglutination activity was inhibited by glycoproteins, such as PSM and asialo-PSM, but not by any of the monosaccharides tested. The activity was stable between 4 °C and 50 °C. Significant ADL activity was observed between pH 4–5. The lectin reaction did not depend on the presence of the divalent cation Ca^{2+} or Mg^{2+}. The N-terminal ADL sequence was determined to be VGYTVGATPM. The lectin exhibited antibacterial activity, involving growth and respiration inhibition in *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Bacillus subtilis* and *Shewanella* sp. Furthermore, ADL showed inhibition activity against the yeast *Saccharomyces cerevisiae*. These findings suggest that ADL plays an important role in the innate immunity of *A. davidianus* on the body surface.

1. Introduction

The giant salamander, *Andrias davidianus*, is a rare amphibian in China. It lives in clear water in mountain rivulets without environmental pollution (Lan et al., 1990). Since the 1980s, artificial *A. davidianus* cultures have been developing in many provinces of China (Jiang et al., 2011). Many *A. davidianus* farms have been established and developed in artificial streams. In recent years, with the rapid growth of *A. davidianus* aquaculture, *A. davidianus* diseases have been reported frequently, and these diseases do serious damage to the cultures. To address this issue, numerous studies have focused on *A. davidianus* skin.

Amphibian skin is a defense organ that fulfills the functions of antimicrobial defense, anti-infection and anti-oxidation (Clarke, 1997; Li et al., 2007; Yang et al., 2009). The skin gland secretion of *A. davidianus* is a source of defense molecules with biological activities. In particular, numerous antimicrobial peptides, phospholipase A_2 and proteolytic enzymes are secreted from *A. davidianus* skin (Wang et al., 2011; Guo et al., 2012). Recently, the skin secretions of *A. davidianus* have been investigated in our laboratory, and glycopeptides have been prepared and characterized (Jin et al., 2011; Feng et al., 2012). However, *A. davidianus* skin components have not been well studied.

Lectins are widely distributed among plants, bacteria and animals, including amphibians. Lectins are one of the important pattern-recognition proteins that have been described as playing a role in processes as diverse as self-defense, parasitism and symbiosis (Jing et al., 2011). They can bind or immobilize microorganisms through agglutination or limit pathogen infection (Holmskov et al., 2003). Unfortunately, there are limited data and literature concerning lectins from amphibian skin secretions compared to that concerning their other organs. For instance, an egg lectin (13.5 kDa) from *Rana japonica* was isolated by gel filtration and successive ion-exchange chromatography on diethylaminoethyl cellulose and carboxymethylcellulose columns (Sakakibara et al., 1979). A β-galactoside binding lectin (30 kDa) from *Bufo arenarum* oocytes was isolated by salt extraction and affinity chromatography, and it was partially characterized with data on

* Correspondence to: W. Li, College of Food Science and Engineering, Dalian Ocean University, Dalian 116023, PR China. Tel.: +86 411 84763553; fax: +86 411 84763508.
** Correspondence to: P. Lukyanov, G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Science, Vladivostok 690022, Russia.
E-mail addresses: paluk@mail.ru (P.A. Lukyanov), aisingioro@hotmail.com (W. Li).
[1] Both authors contributed equally.

http://dx.doi.org/10.1016/j.cbpc.2014.08.008
1532-0456/

its amino acid content and physico-chemical characteristics (Fink de Cabutti et al., 1987). A β-galactoside-binding lectin was isolated and characterized from *Rana catesbeiana* frog eggs (Ozeki et al., 1991). Two β-galactoside-binding lectins (50 and 56 kDa) from the *B. arenarum* skin were isolated and characterized; they showed strong bacteriostatic activity against Gram-negative bacteria (*Escherichia coli* K124100 and wild strains of *E. coli* and *Proteus morganii*) and Gram-positive bacteria (*Enterococcus faecalis*) (Riera et al., 2003). Odorranalectin, a small peptide lectin that recognizes L-fucose, was purified and characterized from *Odorrana grahami* frog skin secretions. This lectin is composed of only 17 amino acid residues (YASPKCFRYPNGVLACT) and contains a single disulfide bridge (Li et al., 2008). Although lectins are found in various organs, the search for new lectins from amphibian skin secretions remains a problem. Studying the properties and functions of lectins from *A. davidianus* skin secretions is a promising endeavor for understanding innate immune defense and for curing related diseases.

In this report, we describe the purification, characterization, carbohydrate specificity and antibacterial activity of a new Ca^{2+}-independent mucin-binding lectin from the giant salamander *A. davidianus*.

2. Materials and methods

2.1. Materials

Monosaccharides were obtained from Merck (Darmstadt, Germany). PSM, porcine stomach mucin (type III), albumin (egg white), thyroglobulin and trypsin were purchased from Sigma Chemical (USA). Human erythrocytes were obtained as outdate red cell concentrates from the Center of Blood Utilization (Dalian). Sephadex G-100 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The TSK gel G3000PW_{XL} column was purchased from TOSOH (Japan). The standard proteins used for apparent molecular mass estimation by SDS-PAGE and HPLC were purchased from Beijing Solarbio Science and Technology Company (Beijing, China).

Gram-positive bacterial strains (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923) and Gram-negative bacterial strains (*E. coli* ATCC 35218, *Clostridium perfringens* ATCC 13124 and *Shewanella* sp.) were used. All strains were kindly provided by the Liaoning Entry–Exit Inspection and Quarantine Bureau. The yeast *Saccharomyces cerevisiae* was obtained from a commercial store (Angel Yeast Co., Ltd., China). The affinity sorbent for crosslinking albumin with PSM was prepared according to the method described previously (Kowal and Parsons, 1980; Belogortseva et al., 1994).

2.2. A. davidianus skin secretion collection

Five 4-year-old, sexually mature giant salamanders (*A. davidianus*) were obtained from Zhangjiajie Jinchi Giant Salamander Biological Technology Company Ltd. (Zhangjiajie city, Hunan province of China). Their use in this research was approved by the Aquatic Wild Animal Researching License of Hunan province (2011-021). Both male and female ($n = 5$, 2 males, 3 females) animals (2-3 kg in mass) were selected randomly for skin secretions. Animals were washed with distilled water to remove contaminants from their skin. Then, the surface was stimulated by pulse durations of 1–2 min with a maximum stimulus strength of 20 V (Tyler et al., 1992). A milky skin secretion was collected and centrifuged at 1200 *g* for 20 min. The supernatant was lyophilized to yield a powder. The powder was stored at 4 °C.

2.3. ADL isolation and purification

100 mg of the powdered sample was suspended in 2 mL of 0.01 M Tris–HCl buffer (TB), pH 7.4, and then centrifuged at 1200 *g* for 20 min. The clear supernatant was applied to a PSM-crosslinked albumin column (3 × 11 cm), which was previously equilibrated and eluted with TB. After the elution of unbound proteins in TB, adsorbed proteins were eluted with 1.5 M NaCl in TB. The protein concentration and hemagglutination titer of each fraction were measured. Fractions containing hemagglutination activity were pooled and further purified by gel filtration on Sephadex G-100 (2.5 × 96 cm) equilibrated with 0.1 M TB, pH 7.4, containing 0.15 M NaCl (TBS). The column was eluted with the same buffer at 10 mL/h, and fractions showing hemagglutination activity were dialyzed against water and lyophilized to yield a powder (6 mg). 50 μg of the purified sample was suspended in 20 μL of 0.01 M TB, pH 7.4, and then centrifuged at 8000 rpm for 20 min. The clear supernatant was additionally purified by high performance liquid chromatography (HPLC) on a G3000PW_{XL} column (7.8 mm × 30 cm). The column was washed with TB.

2.4. Molecular mass measurement of the purified ADL and its subunits

The molecular mass of the purified ADL subunit was measured by SDS-PAGE using a 15% polyacrylamide separation gel and a 4% polyacrylamide stacking gel (Laemmli, 1970). The ADL molecular mass was determined by SDS-PAGE in the presence and absence of dithiothreitol. ADL reduction was performed by heating at 100 °C for 5 min in sample buffer containing 2% SDS and 2.5% dithiothreitol. Gels were calibrated using the following standard proteins: phosphorylase B (94,000), BSA (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and alpha-lactalbumin (14,400). Proteins were stained with Coomassie brilliant blue.

The molecular mass of the purified native lectin was measured by passing it through a G4000PW_{XL} column (7.8 mm × 30 cm) in 0.01 M TBS, pH 7.4. The standard proteins used were rabbit muscle phosphorylase B (97,000), albumin bovine V (68,000), albumin egg (45,000) and trypsin (23,300).

The molecular masses were further investigated by MALDI-TOF mass spectrometry. The measurements were performed on a 4800 Proteomics Analyzer time-of-flight mass spectrometer (Applied Biosystems, USA) at the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

2.5. Hemagglutination assay

To assay the hemagglutination activity, ADL was serially diluted 2-fold with 0.01 M TBS (25 μL) into microtiter U-plates. To each well, an equal volume of 2% human B type erythrocyte suspension was added, and the mixture was agitated. The hemagglutination was visually evaluated after 30 min (Belogortseva et al., 1998b).

For the hemagglutination inhibition assay, aqueous solutions of various substances were serially diluted 2-fold with TBS. ADL (25 μL, 4 doses of agglutinated material) and a 2% erythrocyte suspension (25 μL) were added to each sample (25 μL) successively. The obtained mixture was gently stirred by pipette and incubated for 1 h. The minimal concentration of each substance required for complete inhibition was determined.

2.6. Effect of divalent cations and pH

ADL was dialyzed for 24 h against 0.1 M TBS, pH 7.4, containing 50 mM $EDTANa_2$ or 50 mM $CaCl_2$. Human B type erythrocytes were used as indicator cells.

The ADL pH dependence was determined by preincubating the samples with different pH buffers for 1 h at 25 °C as follows: 0.02 M sodium acetate/acetic acid, pH 3–5; 0.02 M sodium phosphate/HCl, pH 6–7; 0.02 M Tris/HCl, pH 8–8.5; and 0.02 M glycine/NaOH, pH 9–10. The samples were subsequently dialyzed against 0.1 M PBS, pH 7.8, and the agglutination activity was assessed using B type erythrocytes (Belogortseva et al., 1998b).

2.7. Amino acid analysis

Purified ADL (1.0 mg mL^{-1}) was hydrolyzed under argon in a sealed tube with 6 M HCl at 100 °C for 24 h. The ADL amino acid composition was determined with a Hitachi 835 amino acid analysis system.

2.8. Protein and carbohydrate contents, amino acid sequence analysis

The ADL protein content was determined according to the Lowry method (Lowry et al., 1951) using crystalline bovine serum albumin as the standard protein. The sugar content was estimated by the phenol-sulfuric acid method using D-glucose as the standard (Dubois et al., 1956).

The N-terminal amino acid sequence was determined using a PPSQ-33A protein sequencer-N (Shimadzu, Japan) at the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

2.9. Antibacterial assay

The ADL antibacterial activity was determined using *B. subtilis*, *S. aureus*, *E. coli*, *C. perfringens* and *Shewanella* sp. cultures by the agar disc diffusion method. The test was performed in sterile Petri dishes (90 mm diameter) containing LB agar medium. A suspension of the tested microorganism (0.1 mL of 10^7 CFU/mL) was spread on the LB agar medium. ADL absorbed on sterile paper discs (10 μL per disc with a 6 mm diameter) was placed on the surface of the previously inoculated medium (10 μg per Petri dish). Every dish was incubated at 37 °C for 24 h, followed by measuring the inhibition zone diameter (expressed in mm). The scale of measurement was as follows: >16 mm inhibition zone was strongly inhibitory; 11–16 mm inhibition zone was moderately inhibitory; 7–11 mm was weakly inhibitory; and <7 mm inhibition zone was not inhibitory.

2.10. ADL respiration inhibition test

E. coli, *B. subtilis*, *C. perfringens*, *S. aureus* and *Shewanella* sp. were inoculated into LB medium and cultured overnight at 36 °C with shaking at 120 rpm. The bacteria were collected by centrifugation (4 °C, 20 min, 1200 *g*). The pelleted bacterial cells were washed three times in 0.1 M PBS, pH 7.4, containing 0.15 M NaCl and diluted in the same PBS to a final OD_{600} of 1.0.

A solution of 15 mL of PBS, 1 mL of 1% glucose and 1 mL of bacterial suspension was stirred vigorously for 5 min in a reactor that was sealed with Parafilm. During the respiration inhibition test, each reactor was continuously stirred. Dissolved oxygen (DO) was measured with a DO probe at 1 min intervals for 10 min (mg O_2 L^{-1}). The controlled respiration rates (R_0) in mg O_2 L^{-1} min^{-1} were determined from the slope of the linear portion of the DO vs. time curve (Wang et al., 2008).

The controlled solution was added either to 100 μL of sodium phosphate (50 mg mL^{-1}), malonic acid (50 mg mL^{-1}), iodoacetic acid (50 mg mL^{-1}) or ADL (12.5 mg mL^{-1}) or to a combination of 100 μL of sodium phosphate and 100 μL of ADL, 100 μL of malonic acid and 100 μL of ADL or 100 μL of iodoacetic acid and 100 μL of ADL. The inhibitor respiration rates (R_I) were determined from the slope of the linear portion of the DO vs. time curve. The percent inhibition was obtained according to Eq. (1) (against the control average):

$$I_R = (R_0 - R_I)/R_0 \times 100 \quad (1)$$

where R_I is the respiration rate of the inhibitor in μmol O_2 g^{-1} min^{-1}; R_0 is the control respiration rate in μmol O_2 g^{-1} min^{-1}; and I_R is the respiration rate of the inhibitor against bacteria.

Superposition of the inhibitory rate was obtained according to Eq. (2) (against the typical average):

$$R_R = (R_L - R_{LI})/R_L \times 100 \quad (2)$$

where R_L is the respiration rate of ADL in μmol O_2 g^{-1} min^{-1}; R_{LI} is the respiration rate of the combination of typical inhibitors and ADL in μmol O_2 g^{-1} min^{-1}; and R_R is the superposition of the inhibitory rate (Wang et al., 2008).

2.11. Test for ADL anti-fungal activity

The cultivation medium consisted of D-glucose (150 g L^{-1}), yeast extract (5 g L^{-1}) and peptone (10 g L^{-1}). Cell growth was determined by plate counting in some cases. Samples were withdrawn throughout fermentation and diluted appropriately in dilution medium (Bely et al., 2008).

Yeast cells grew in 100 mL of cultivation medium to an exponential phase for 2 h after the number of cells had reached approximately 6×10^6 cells mL^{-1}. These cells were used to initiate growth in the other media used in this study at a 1% v/v inoculum. All ethanol fermentation studies were performed at 150 rpm in rotary-agitated 250 mL Erlenmeyer flasks containing 100 mL of cultivation medium at 29 °C for 24 h. ADL inhibition was studied in the cultivation medium by adding different concentrations (0.0684, 1.171 and 8.55 mg L^{-1} (w/v)) of ADL. Samples were withdrawn at 24 h. After 24 h, 60 μL of cultured solution was removed to measure the OD at 600 nm.

2.12. Statistical analysis

Each experiment was performed in triplicate. Values are presented as the means ± standard deviation.

3. Results

3.1. ADL purification

For ADL purification, affinity chromatography techniques are the most efficient isolation methods. ADL was purified by affinity chromatography on a PSM-crosslinked albumin column. The elution profile is presented in Fig. 1. The specifically bound fraction was eluted with TB containing 1.5 M NaCl. ADL was further purified by gel filtration on Sephadex G-100 (Fig. 2). The final step of purification was by HPLC on a TSK gel G3000PW_{XL} column (Fig. 3). ADL migrated as a symmetrical peak by gel filtration, and the hemagglutination activity was exactly coincident with the protein content (data not shown). The purified lectin was eluted from the TSK gel G4000PW_{XL} column at (Ve − Vo) / Vo =

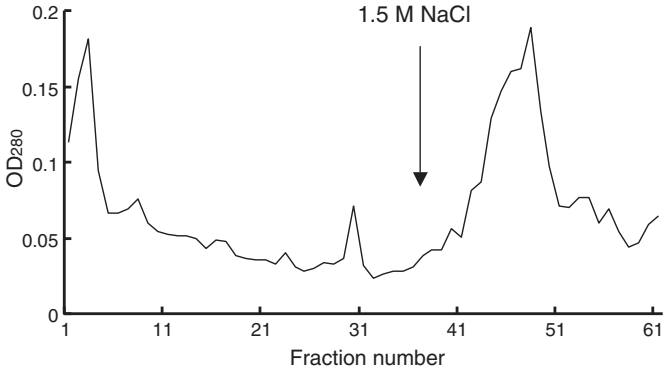

Fig. 1. Affinity chromatography of the *A. davidianus* skin secretion on a PSM-crosslinked albumin column (3 × 11 cm) equilibrated and eluted with TB. The bound fraction was eluted with 1.5 M NaCl in TB.

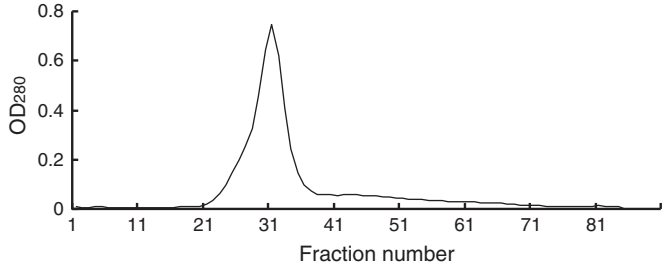

Fig. 2. Gel chromatography of ADL on Sephadex G-100 (2.5 × 96 cm).

4.76 (Fig. 4). The molecular mass of ADL estimated from HPLC had an apparent about 68 kDa.

The ADL was analyzed by SDS-PAGE and found to migrate as a major intense band with a relative molecular mass of approximately 17 kDa (Fig. 5, lane 1). Reduction with dithiothreitol led to the appearance of an 8.5 kDa band (Fig. 5, lane 3). This finding indicates the presence of a disulfide bond and two subunits with identical molecular masses.

The ADL molecular mass was further determined by MALDI-TOF mass spectrometry. The ADL spectrum contained peaks corresponding to singly charged $(M + H)^+$ molecular ions from the subunit at *m/z* 8.5 kDa, while the peak at *m/z* 17 kDa corresponded to the subunit dimer (Fig. 6).

In summary, the molecular mass determination by SDS-PAGE and MALDI-TOF mass spectrometry showed that ADL has two subunits with a molecular mass of 17 kDa (2 × 8.5 kDa). The ADL N-terminal amino acid sequence was determined to be VGYTVGATPM. According to the hemagglutination assay, ADL does not require the divalent cations Ca^{2+} and Mg^{2+} for lectin activity (data not shown). The ADL lectin activity was stable between 4 °C and 50 °C (Fig. 7 A). Significant ADL hemagglutination activity was observed between pH 4–5 (Fig. 7 B).

3.2. Amino acid composition and carbohydrate content

As shown in Table 1, purified ADL contains relatively high amounts of the apolar amino acids Gly, Pro, Val and Leu, and essential proportion of polar charged amino acids His, Arg and apolar amino acid Met. The total carbohydrate content was 1%.

3.3. Carbohydrate-binding specificity

The ADL carbohydrate-binding specificity was examined by a hemagglutination inhibition test. The ADL hemagglutination activity was not inhibited by any of the monosaccharides or disaccharide examined,

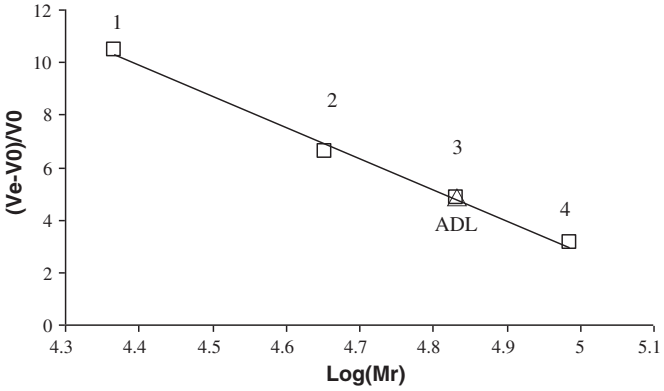

Fig. 4. Estimation of the ADL molecular mass by HPLC on the TSK gel G4000PW$_{XL}$ column. 1, Trypsin (23,300); 2, Albumin egg (45,000); 3, Albumin bovine V (68,000); 4, Rabbit muscle phosphorylase B (97,000).

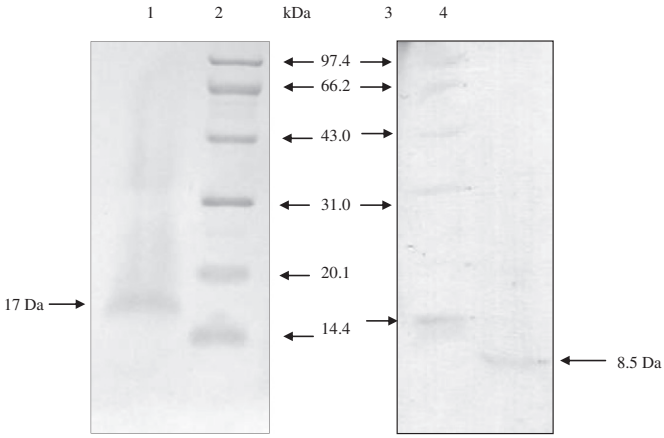

Fig. 5. SDS-PAGE. Lane 1-ADL, Mr ~17,000. Lane 2, 3-marker proteins: Rabbit phosphorylase b (97,400), bovine serum albumin (66,200), rabbit actin (43,000), bovine carbonic anhydrase (31,000), trypsin inhibitor (20,100), hen egg white lysozyme (14,400). Lane 4-ADL treated with dithiothreitol.

whereas it was inhibited by several glycoproteins (Table 2). Among the glycoproteins, asialo-PSM was the most effective inhibitor.

3.4. Antibacterial activity

The ADL antibacterial activity results are shown in Table 3. ADL exhibited strong antibacterial activity against *E. coli*. ADL also exhibited

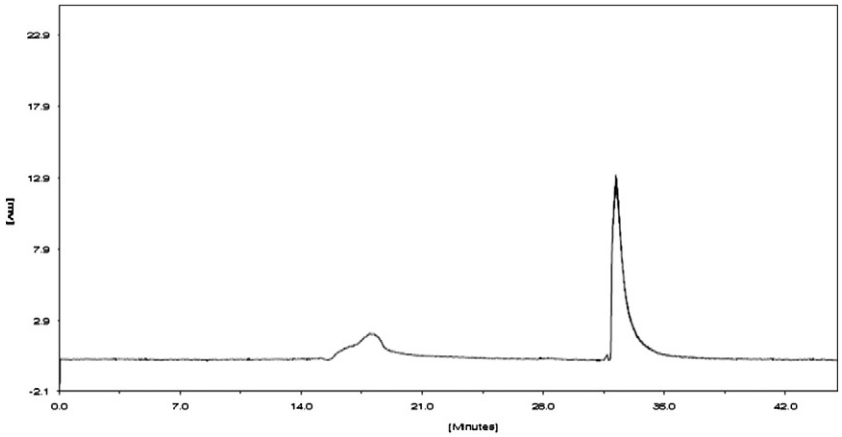

Fig. 3. HPLC of purified ADL on a G3000PW$_{XL}$ column (7.8 mm × 30 cm).

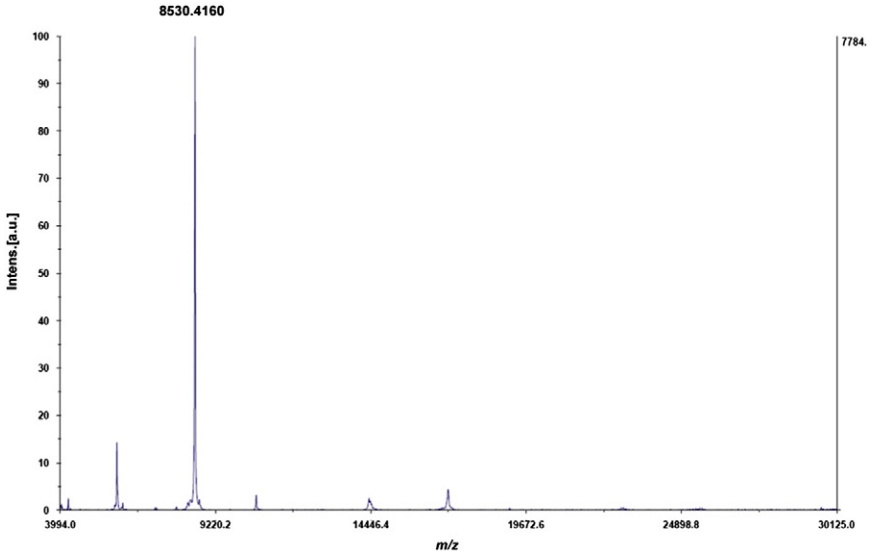

Fig. 6. MALDI-TOF mass spectrum of ADL. The mass spectrum was obtained on a 4800 Proteomics Analyzer time-of-flight mass spectrometer (Applied Biosystems, USA).

antibacterial activity against *Enterobacter aerogenes*, *S. aureus*, *B. subtilis* and *Shewanella* sp.

The respiration inhibition of *E. coli*, *E. aerogenes*, *S. aureus*, *B. subtilis* and *Shewanella* sp. was determined by high-resolution respirometry (dissolved oxygen meter 8401, AZ Instrument Corp., Taiwan). Malonic acid, iodoacetic acid and sodium phosphate are three typical inhibitors of respiratory metabolism and affect the Embden–Meyerhof Pathway (EMP), tricarboxylic acid cycle (TCAC) and Hexose Monophosphate Pathway (HMP), respectively (Wang et al., 2008). When two inhibitors block different pathways, the superposition of the inhibitory rates increases greatly; however, if they inhibit the same pathway, then the rate increase is weak. ADL inhibited the respiration of *E. coli*, *E. aerogenes*, *S. aureus*, *B. subtilis* and *Shewanella* sp., and the inhibition rates are 23.7 ± 4.3%, 13.9 ± 5.5%, 59.2 ± 1.4%, 46.2 ± 0.7% and 31.8 ± 4.6%, respectively (Table 4). These rates indicated that ADL inhibited the growth of *S. aureus* more than the other four bacteria. For *E. coli*, the inhibitory superposition rates of malonic acid, iodoacetic acid and sodium phosphate to ADL were 16.1 ± 1.6%, 69.9 ± 0.5% and 25.1 ± 1.5%, respectively, which indicted that ADL inhibited the same pathway as malonic acid, i.e., TCAC. For *E. aerogenes*, the inhibitory superposition rates of malonic acid, iodoacetic acid and sodium phosphate to ADL were 53.2 ± 3.7%, 80.0 ± 0.6% and 6.8 ± 2.5%, respectively, which indicted that ADL inhibited the same pathway as sodium phosphate, i.e., HMP. For *S. aureus*, the inhibitory superposition rates of malonic acid, iodoacetic acid and sodium phosphate to ADL were 10.0 ± 2.1%, 58.0 ± 1.7% and 44.0 ± 1.9%, respectively, which indicted that ADL inhibited the same pathway as malonic acid, i.e., TCAC. For *B. subtilis*, the inhibitory superposition rates of malonic acid, iodoacetic acid and sodium phosphate to ADL were 64.3 ± 1.7%, 41.7 ± 3.3% and 12.5 ± 2.6%, respectively, which indicted that ADL inhibited the same pathway as sodium phosphate, i.e., HMP. For *Shewanella* sp., the inhibitory superposition rates of malonic acid, iodoacetic acid and sodium

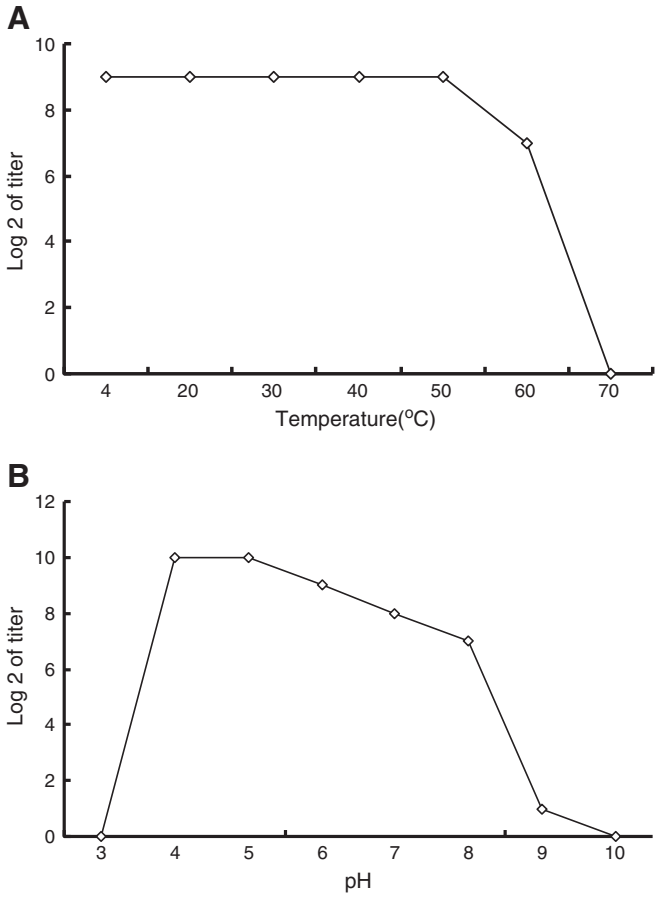

Fig. 7. General properties of the ADL. The effects of temperature (A) and pH (B) on hemagglutination activity.

Table 1
ADL amino acid composition.

Amino acid	Residues/100
Asx	4.76
Thr	7.05
Ser	5.62
Glx	5.25
Pro	9.02
Gly	17.88
Ala	4.83
Val	7.16
Ile	7.14
Leu	8.25
Tyr	5.26
Phe	5.27
His	1.79
Lys	7.22
Arg	2.07
Met	1.32

Cys, Trp — not determined.

Table 2
Hemagglutination inhibition test with carbohydrates and glycoproteins.

Carbohydrate and glycoprotein	Minimum inhibitory concentration (mM or μg mL^{-1})
Carbohydrate (mM)	
D-Glucose	–
D-Galactose	–
D-Mannose	–
N-acetyl-D-glucosamine	–
N-acetyl-D-galactosamine	–
Fructose	–
Sorbose	–
Xylose	–
Cellobiose	–
Glycoprotein (μg mL^{-1})	
Mucin from porcine stomach (PSM)	0.078
Asialo-PSM	0.008
Thyroglobulin	–

Dashes indicate no inhibitory activity at 100 mM monosaccharide.

Table 3
ADL antibacterial activity.

	E. coli	*S. aureus*	*B. subtilis*	*E. aerogenes*	*Shewanella* sp.
ADL	+++	++	++	+	+
Penicillin	+++	+++	++	++	++

(–) No activity, (+) weak activity is 7–11 mm, (++) moderate activity is 11–16 mm, (+++) high activity is >16 mm.

phosphate to ADL were 31.5 ± 2.9%, 59.2 ± 1.3% and 14.3 ± 3.2%, respectively, which indicted that ADL inhibited the same pathway as sodium phosphate, i.e., HMP.

3.5. ADL suppresses yeast growth

To investigate the anti-fungal activity of ADL, its suppressive effect on yeast cell growth was examined. The OD_{600} of yeast suspension with different ADL concentrations (0.0684, 1.171 and 8.55 mg L^{-1} (w/v)) was obviously different from that of the control (Fig. 8). The number of yeast cells declined with increasing concentrations of ADL, indicating that this lectin suppresses *S. cerevisiae* growth.

4. Discussion

As sources of lectins, amphibian skin secretions have not been widely investigated in comparison with other organs (eggs and oocytes). Our interest in skin secretion lectins arose from recent reports on the isolation and characterization of two β-galactoside binding lectins (50 and 56 kDa) from the skin of *B. arenarum* that have bacteriostatic activity against Gram-negative bacteria (*E. coli* K124100 and wild strains of *E. coli* and *P. morganii*) and Gram-positive bacteria (*E. faecalis*) (Riera et al., 2003). Additionally, the L-fucose specific lectin from the skin of another amphibian (*O. grahami*) has extremely low toxicity and immunogenicity in mice (Li et al., 2008).

A new lectin secreted from *A. davidianus* skin was isolated by affinity chromatography on PSM crosslinked albumin and purified by gel

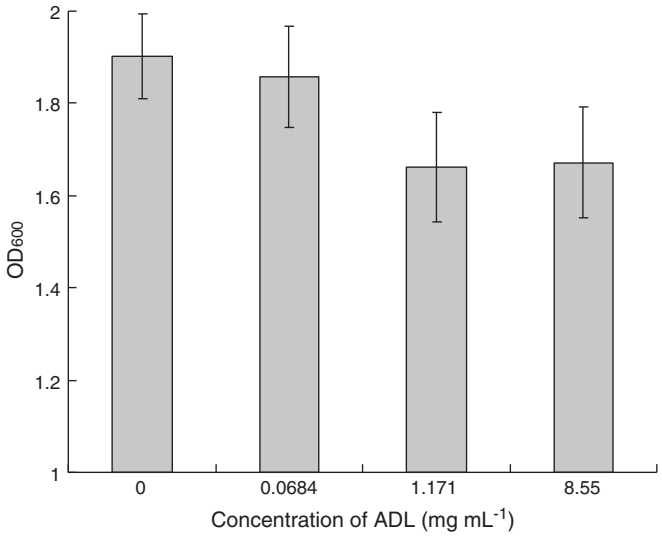

Fig. 8. ADL growth suppressive activity against yeast cells.

filtration on Sephadex G-100 and HPLC on TSK gel G3000PW_{XL}. The molecular weight of ADL was estimated by SDS-PAGE, HPLC and MALDI-TOF mass spectrometry. The results indicate that the native molecular weight of ADL is 68 kDa and that it is composed of two identical 8.5 kDa subunits. The native ADL is a homotetrameric glycoprotein that has two identical subunits that are connected with disulfide bonds and are organized as a tetramer. Such structural organization is very rare, though similar tetrameric structures have been described for lectins from the sea worm *Serpula vermicularis* (Molchanova et al., 2007), the white shrimp *Litopenaeus setiferus* (Alpuche et al., 2005) and the tropical sponges *Aplysina archeri* and *Aplysina lawnosa* (Miarons and Fresno, 2000). The ADL hemagglutination activity was independent of divalent cations. Significant ADL hemagglutination activity was observed between pH 4-5. The amino acid composition differs from that of lectins isolated from the skin mucus of fish, where ADL is rich in apolar amino acids, such as Gly, Pro, Val and Leu (Tsutsui et al., 2003, 2007).

The results of the hemagglutination inhibition study suggest that the topography of the ADL-combining sites is significantly different from other known mucin-binding lectins (Belogortseva et al., 1998a,b; Banerjee et al., 2004). Among the native and asialo-glycoproteins tested in the hemagglutination inhibition assays, asialo-PSM was the best inhibitor. The carbohydrate side chains of PSM are *O*-glycosidically linked through GalNAc to Ser or Thr of the protein core (Gerken et al., 1998). There are 12 carbohydrate side chains present, which are composed of one to five sugar residues, with Galβ1-3 GalNAc α-*O*-Ser/Thr as the carbohydrate core region. The high PSM potency may be attributed to the presence of Galβ1-3 GalNAc α-*O*-Ser/Thr, where GalNAc is substituted at C 3 by a Gal residue. The carbohydrate chain can be masked by N-glycolylneuraminic acid or sialic acid (NeuNGl). The inhibitory ability of asialo-PSM was 10-fold greater than PSM. Most likely, NeuGl residues, which have negative charges, interfere with the interaction of PSM with ADL. The ADL hemagglutination activity was strongly

Table 4
Effect of ADL on bacterial respiration inhibition. Values are means ± SD (n = 3).

Inhibitor	*E. coli*		*E. aerogenes*		*S. aureus*		*B. subtilis*		*Shewanella* sp.	
	I_R (%)	R_R (%)	I_R (%)	R_R (%)	I_R (%)	R_R (%)	I_R (%)	R_R (%)	I_R (%)	R_R (%)
ADL	23.7 ± 4.3		13.9 ± 5.5		59.2 ± 1.4		46.2 ± 0.7		31.8 ± 4.6	
Malonic acid	61.9 ± 1.1	16.1 ± 1.6	89.7 ± 1.2	53.2 ± 3.7	5.7 ± 2.0	10.0 ± 2.1	62.3 ± 2.4	64.3 ± 1.7	49.9 ± 3.6	31.5 ± 2.9
Iodoacetic acid	85.7 ± 0.2	69.9 ± 0.5	32.5 ± 6.1	80.0 ± 0.6	82.8 ± 1.0	58.0 ± 1.7	59.6 ± 1.9	41.7 ± 3.3	33.3 ± 2.4	59.2 ± 1.3
Sodium phosphate	20.0 ± 1.4	25.1 ± 1.5	31.3 ± 6.4	6.8 ± 2.5	8.3 ± 2.9	44.0 ± 1.9	22.6 ± 4.0	12.5 ± 2.6	22.3 ± 3.1	14.3 ± 3.2

inhibited by PSM bearing mucin-type *O*-glycans, such as the lectin from *Crenomytilus grayanus* (Belogortseva et al., 1998b) and the lectin from the sponge *Craniella australiensis* (Xiong et al., 2006).

After the pathway of glucose oxidative metabolism was inhibited, the activities necessary for life were interrupted. EMP, TCAC and HMP are glucose degradation pathways. TCAC could provide massive energy and compounds for the synthesis of other biomacromolecules, such as lipids and proteins. ADL inhibited TCAC in *E. coli* and *S. aureus* as well as HMP in *E. aerogenes*, *Shewanella* sp. and *B. subtilis*. These results are consistent with ADL antibacterial activity, i.e. ADL can inhibit *E. coli* and *S. aureus* more efficiently than *E. aerogenes* and *Shewanella* sp.

Additionally, the inhibitory effect had no relationship with the type of bacteria, i.e. Gram-positive or Gram-negative. ADL showed greater inhibitory effects on Gram-negative bacteria, such as *E. coli*, than Gram-positive bacteria, such as *S. aureus* and *B. subtilis*. However, ADL had a greater inhibitory effect on Gram-positive bacteria, like *S. aureus* and *B. subtilis*, than Gram-negative bacteria, like *E. aerogenes* and *Shewanella* sp. ADL could also suppress yeast cell growth, which suggests that ADL is involved in self-defense against fungi and bacteria.

Microbial infection is the leading cause of disease in *A. davidianus* (Jiang et al., 2011). Through evolution, antimicrobial components, including lectins, have given rise to counterparts in microorganisms (Li et al., 2008). ADL participates in the *A. davidianus* host defense. The lectin may inhibit the growth of microorganisms in the mucus, some of which may serve as a nutrient to the microorganism (Tasumi et al., 2002). ADL also revealed the *A. davidianus* immune state.

Acknowledgments

The work was supported by grants from the Natural Science Foundation of China (31071612) and the Marine Public Welfare Research Project (201205022-7).

References

Alpuche, J., Pereyra, A., Agundis, C., Rosas, C., Pascual, C., Slomianny, M.C., Vazquez, L., Zenteno, E., 2005. Purification and characterization of a lectin from the white shrimp *Litopenaeus setiferus* (*Crustacea decapoda*) hemolymph. Biochim. Biophys. Acta 1724, 86–93.

Banerjee, S., Chaki, S., Bhowal, J., Chatterjee, B.P., 2004. Mucin binding mitogenic from freshwater Indian gastropod *Belamyia bengalensis*: purification and molecular characterization. Arch. Biochem. Biophys. 421, 125–134.

Belogortseva, N.I., Ovodova, R.G., Moroz, S.V., Odintsova, N.A., Yermak, A.V., Ovodov, Y.S., 1994. Isolation and characterization of a structurally unusual lectin from ascidian *Didemnum ternatum* (DTL). Bioorg. Khim 20, 975–983.

Belogortseva, N., Molchanova, N., Glazunov, V., Evtushenko, E., Luk'ynov, P., 1998a. N-Acetylglucosamine-specific lectin from the ascidian *Didemnum ternatanum*. Biochim. Biophys. Acta 1380, 249–256.

Belogortseva, N.I., Molchanova, V.I., Kurika, A.V., Skobun, A.S., Glazkova, V.E., 1998b. Isolation and characterization of new GalNAc/Gal-specific lectin from the sea mussel *Crenomytilus grayanus*. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 119, 45–50.

Bely, M., Storckle, P., Masneuf-Pomarede, I., Dubourdieu, D., 2008. Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. Int. J. Food Microbiol. 122, 312–320.

Clarke, B.T., 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol. Rev. Camb. Philos. Soc. 72, 365–379.

Dubois, M., Gilles, K.A., Hamilton, I.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Feng, X., Qu, M., Yu, X., Li, W., Wang, W., Tong, C., Kong, L., Jin, Q., 2012. Study on properties of glycopeptides from *Andrias davidianus*. Chin. Sci. Technol. Food Ind. 33, 128–131.

Fink de Cabutti, N.E., Caron, M., Joubert, R., Elola, M.T., Bladier, D., Herkovitz, J., 1987. Purification and some characteristics of a β-galactoside binding soluble lectin from amphibian ovary. FEBS Lett. 223, 330–334.

Gerken, T.A., Owens, C.L., Pasumarthy, M., 1998. Site-specific core 1 *O*-glycosylation pattern of the porcine submaxillary gland mucin tandem repeat: evidence for the modulation of glycan length by peptide sequence. J. Biol. Chem. 273, 26580–26588.

Guo, W., Ao, M., Li, W., Wang, J., Yu, L., 2012. Major biological activities of the skin secretion of the Chinese giant salamander, *Andrias davidianus*. Z. Naturforsch. C 67, 86–92.

Holmskov, U., Thiel, S., Jensenius, J.C., 2003. Collectins and ficolins: humoral lectins of the innate immune defense. Annu. Rev. Immunol. 21, 547–578.

Jiang, Y.L., Zhang, M., Jing, H.L., Gao, L.Y., 2011. Isolation and characterization of an iridovirus from sick giant salamander (*Andrias davidianus*). Chin. J. Virol. 27, 274–282.

Jin, Q., Wei, F., Tong, C., Li, W., Kong, L., 2011. HPLC analysis and antioxidative activity of glycopeptides from *Andrias davidianus*. Chin. J. Beijing Univ. Agric. 26, 27–29.

Jing, X., Espinosa, E.P., Perrigault, M., Allam, B., 2011. Identification, molecular characterization and expression analysis of a mucosal C-type lectin in the eastern oyster, *Crassostrea virginica*. Fish Shellfish Immunol. 30, 851–858.

Kowal, R., Parsons, R.G., 1980. Stabilization of proteins immobilized on Sepharose from leakage by glutaraldehyde crosslinking. Anal. Biochem. 102, 72–76.

Laemmli, U.K., 1970. Cleavage of structure proteins during assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lan, S.C., Li, D.F., Jiang, J.C., 1990. Call and skin glands secretion induced by stimulation of midbrain in urodele (*Andrias davidianus*). Brain Res. 528, 159–161.

Li, J., Xu, X., Xu, C., Zhou, W., Zhang, K., Yu, H., Zhang, Y., Zheng, Y., Rees, H.H., Lai, R., Yang, D., Wu, J., 2007. Anti-infection peptidomics of amphibian skin. Mol. Cell. Proteomics 6, 882–894.

Li, J., Wu, H., Hong, J., Xu, X., Yang, H., Wu, B., Wang, Y., Zhu, J., Lai, R., Jiang, X., Lin, D., Prescott, M.C., Rees, H.H., 2008. Odorranalectin is a small peptide lectin with potential for drug delivery and targeting. PLoS ONE 3, 1–10.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randae, R.I., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Miarons, P.B., Fresno, M., 2000. Lectins from tropical sponges: purification and characterization of lectins from genus *Aplysina*. J. Biol. Chem. 275, 29283–29289.

Molchanova, V., Chikalovets, I., Chernikov, O., Belogortseva, N., Li, W., Wang, J., Ou Yang, D.Y., Zheng, Y.T., Lukyanov, P., 2007. A new lectin from the sea worm *Serpula vermicularis*: Isolation, characterization and anti-HIV activity. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 145, 184–193.

Ozeki, Y., Matsui, T., Nitta, K., Kawauchi, H., Takayanagi, Y., Titani, K., 1991. Purification and characterization of β-galactoside binding lectin from frog (*Rana catesbeiana*) eggs. Biochem. Biophys. Res. Commun. 178, 407–413.

Riera, A.S., Daud, A., Gallo, A., Genta, S., Aybar, M., Sanchez, S., 2003. Antibacterial activity of lactose-binding lectins from *Bufo arenarum* skin. Biocell 27, 37–46.

Sakakibara, F., Kawauchi, H., Takayanagi, G., Ise, H., 1979. Egg lectin of *Rana japonica* and its receptor glycoprotein of Ehrlich tumor cells. Cancer Res. 39, 1347–1352.

Tasumi, S., Ohira, T., Kawazoe, I., Suetake, H., Suzuki, Y., Aida, K., 2002. Primary structure and characteristics of a lectin from skin mucus of the Japanese eel *Anguilla japonica*. J. Biol. Chem. 277, 27305–27311.

Tsutsui, S., Tasumi, S., Suetake, H., Suzuki, Y., 2003. Lectins homologous to those of monocotyledonous plants in the skin mucus and intestine of pufferfish, *Fugu rubripes*. J. Biol. Chem. 278, 20882–20889.

Tsutsui, S., Iwamoto, K., Nakamura, O., Watanable, T., 2007. Yeast-binding C-type lectin with opsonic activity from conger eel (*Conger myriaster*) skin mucus. Mol. Immunol. 44, 691–702.

Tyler, M.J., Stone, D.J., Bowie, J.H., 1992. A novel method for the release and collection of dermal glandular secretions from the skin of frogs. J. Pharmacol. Toxicol. Methods 28, 199–200.

Wang, H.-T., Shi, S.-S., Li, Y.-X., Li, H.-Q., Xie, M.-J., 2008. Study on anti-microbial activity of genistein and its mechanism. Acta Nutr. Sin. 30, 403–409.

Wang, L., Li, X., Wang, D., 2011. Antibacterial effect of antimicrobial peptide from skin secretions of *Andrias davidianus* on the wound of *Pseudomonas aeruginosa* infection in mice. West China J. Pharm. Sci. 26, 336–339.

Xiong, C., Li, W., Liu, H., Zhang, W., Dou, J., Bai, X., Du, Y., Ma, X., 2006. A normal mucin-binding lectin from the sponge *Craniella australiensis*. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 143, 9–16.

Yang, H., Wang, X., Liu, X., Wu, J., Liu, C., Gong, W., Zhao, Z., Hong, J., Lin, D., Wang, Y., Lai, R., 2009. Antioxidant peptidomics reveals novel skin antioxidant system. Mol. Cell. Proteomics 8, 571–583.